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Testosterone Induces Up-Regulation Of Mitochondrial Gene Expression In Murine C2C12 Skeletal Muscle Cells Accompanied By An Increase Of Nuclear Respiratory Factor-1 And Its Downstream Effectors

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Running Title: Effect of testosterone on mitochondrial gene expression

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Abstract

The reduction in muscle mass and strength with age, sarcopenia, is a prevalent condition among the elderly, linked to skeletal muscle dysfunction and cell apoptosis. We demonstrated that testosterone protects against H_2O_2-induced apoptosis in C2C12 muscle cells. Here, we analyzed the effect of testosterone on mitochondrial gene expression in C2C12 skeletal muscle cells. We found that testosterone increases mRNA expression of genes encoded by mitochondrial DNA, such as NADPH dehydrogenase subunit 1 (*ND1*), subunit 4 (*ND4*), cytochrome b (*CytB*), cytochrome c oxidase subunit 1 (*Cox1*) and subunit 2 (*Cox2*) in C2C12. Additionally, the hormone induced the expression of the nuclear respiratory factors 1 and 2 (*Nrf-1* and *Nrf-2*), the mitochondrial transcription factors A (*Tfam*) and B2 (TFB2M), and the optic atrophy 1 (OPA1). The simultaneous treatment with testosterone and the androgen receptor antagonist, Flutamide, reduced these effects. H_2O_2-oxidative stress induced treatment, significantly decreased mitochondrial gene expression. Computational analysis revealed that mitochondrial DNA contains specific sequences, which the androgen receptor could recognize and bind, probably taking place a direct regulation of mitochondrial transcription by the receptor. These findings indicate that androgen plays an important role in the regulation of mitochondrial transcription and biogenesis in skeletal muscle.

Keywords: Testosterone; AR; NRF-1; Tfam; mitochondrial biogenesis; mitochondrial genes

Introduction

Testosterone is a key determinant of body composition in male mammals, including human, owing to its effects on muscle and fat mass. The age-related decrease in the levels of serum testosterone in men has been related to the loss of skeletal muscle mass and strength, and the physical performance [Kovacheva 2009; Ferrando 2002; Seidman 2007; Krasnoff 2010], also known as sarcopenia [Cruz-Jentoft et al. 2010]. Testosterone induces the increment in muscle size, related to hypertrophy of muscle fibers and significant increases in myonuclear and satellite cell numbers [Sinha-Hikim et al. 2002, 2003, 2006]. Existing evidence further proposes that exogenous testosterone supplementation results in a faster recovery from hind limb paralysis after injury of sciatic nerve of rat [Brown et al. 1999], and completely prevents apoptosis induced by castration in levator ani muscle cells in rats [Boissonneault et al. 2001]. Although the exact mechanisms underlying the loss of muscle associated with aging are far from being completely clarified, accumulating evidence postulates that an age-related acceleration of muscle cells loss via apoptosis could represent a crucial mechanism responsible for impairment of muscle performance [Dirks & Leeuwenburgh 2002, Dupont-Versteegden 2005]. Of relevance, in our laboratory we demonstrated that testosterone protects against H_2O_2-induced apoptosis in the C2C12 muscle cell line at morphological, physiological and biochemical level [Pronsato et al. 2010, 2012, 2016], having the androgen receptor (AR) an active participation in these events. C2C12 myoblasts bear resemblance to the activated satellite cells that surround the mature myofibers and proliferate and differentiate, taking part of the repair of the tissue when a cellular injury exists [Yoshida et al. 1998] having an vital role in skeletal muscle recovery [La Colla et al. 2015].

The AR, a member of the superfamily of nuclear receptors which comprises a large group of ligand-regulated transcription factors [Katzenellenbogen and Katzenellenbogen 1996], mediates several biological actions of androgens. When AR binds to androgen, the hormone-receptor complex translocates to the nucleus, where the modulation of gene target expression takes place over a period of hours [He et al. 1999; Heinlein and Chang 2002; Wierman 2007].

Scientific evidence accumulated in the last years, suggests the existence of membrane androgen receptors (mAR) triggering rapid non-genomic actions, in various cell types such as macrophages and T cells [Benten et al. 1999a; 1999b], LNCaP [Wang et al. 2008], MCF7 [Kallergi et al. 2007], DU145 [Hatzoglou et al. 2005; Papadopoulou et al. 2008; 2009], C6 [Gatson et al. 2006], PC12 [Alexaki et al. 2006] and VSMC cells [Somjen et al. 2004]. Of relevance, extranuclear organelles have been also proposed as containing the AR. The presence of the AR in mitochondria of mammalian cells including skeletal muscle has been reported [Solakidi et al. 2005; Pronsato et al. 2013]. Biochemical and immunological data provided by our laboratory has supported the non-classical localization of the AR in mitochondria and microsomes of C2C12 skeletal

muscle cells [Pronsato et al. 2013], from where it could be participating in the antiapoptotic effect of testosterone on skeletal muscle [Pronsato et al. 2012]. Thus, non-classical localization of AR from where it can exert non-genomic actions could be possible.

In skeletal muscle, mitochondrial oxidative phosphorylation serve as a major source of energy to meet basic metabolic needs and energy demands during physical activity. Some studies have linked the progressive decline in skeletal mitochondrial function with aging and functional decline, where physical strength declines out of proportion to the loss of muscle mass [Carter et al. 2012; Parise & De Lisio 2010]. To optimize energy production and minimize oxidative damage, mitochondria are engaged in dynamic network exchange through fission and fusion [Bereiter-Hahn & Voth 1994; Schafer & Reichert 2009]. Impairments of these mechanisms of mitochondrial quality control during aging contribute to the age-related increase in tissue oxidative damage and functional decline [Weber & Reichert 2010; Green et al. 2011; Xu et al. 2010; Hofer et al. 2010]. Aging can be precipitately induced by oxidative stress, through the accumulation of reactive oxygen species (ROS) [Renault et al. 2002]. Indeed, aging is related to excessive ROS levels, which provoke an increment in mitochondrial damage [Vasconsuelo et al. 2013]. Mitochondria could produce higher ROS levels in the aged muscle, inactivating satellite cells and thus, contributing thus to the impairment of its restorative function. Deficits in mitochondrial content and quality are often observed in aging muscle, although this topic remains under considerable debate [Carter et al. 2015; Hepple 2014]. Whereas some studies have noted no changes in mitochondria with aging, others have well documented reductions in mitochondrial content [Chabi et al. 2008; Ljubicic and Hood 2009], enhanced ROS emission [Ljubicic et al. 2009], increased apoptotic susceptibility [Gouspillou 2014; Chabi 2008], decreased capacity of calcium retention [Gouspillou et al. 2014], and an impaired signaling for biogenesis [Ljubicic and Hood 2009; Reznick et al. 2007]. Although mitochondrial dysfunction is thought to be associated to aging, how exactly aging influences mitochondrial dysfunction is not well understood.

Mitochondrial biogenesis is an intricate event that involves both mitochondrial proliferation (mitochondrial mass increment) and differentiation (mitochondrial capacities improvement) [Attardi and Schatz 1988], whose modulation needs the coordinated contribution of proteins derived from both nuclear and mitochondrial genomes [Fernandez-Silva et al. 2003]. The mitochondrial genome (16 Kb circular mtDNA) encode some of the subunits of complexes I, III, IV and V of the mitochondrial respiratory chain (MRC) in addition to other proteins, ribosomal and transfer RNAs, involved in mitochondrial activity [Montoya et al. 2006]. Furthermore, mtDNA contains response elements for class I and II receptors, where the AR belongs to [Demonacos et al. 1995, 1996]. In fact, there are evidences that have taken into account that androgens, through the AR, can not only regulate the expression of nuclear genes that

encode for some subunits of the MRC, but also regulate the expression of mitochondrial genes that also encode for subunits of this chain. This could be through a direct way, by binding to the response elements for androgens present in the mtDNA or through an indirect way by regulating the expression of the nuclear genes that encode nuclear or mitochondrial transcription factors involved in the regulation of the expression of these subunits [Psarra et al. 2006; Usui et al. 2014]. The mode by which androgens mediate these events are currently unknown. Studies have assigned the nuclear respiratory factor-1 (NRF-1) transcription factor a key role in the integration of the transcription of nuclear- and mitochondrial-encoded genes [Evans and Scarpulla 1989]. NRF-1 homodimerizes and binds to palindromic NRF-1 sites in the promoters of nuclear-encoded mitochondrial genes [Virbasius et al. 1993]. Target genes of NRF-1 comprise subunits of the five MRC complexes, assembly factors for the respiratory apparatus, parts of the machinery needed for the mtDNA transcription and replication, components of mitochondrial protein import, mitochondrial and cytosolic enzymes of heme biosynthesis, and three crucial mtDNA transcription factors: mitochondrial transcription factor A (Tfam) and mitochondrial transcription factor B types 1 and 2 (TFB1M and TFB2M) [Kelly 2004]. TFAM, TFB1M and TFB2M transcribe the mitochondrial genome, leading to an increment in mitochondrial-encoded subunits of the MRC [Kelly and Scarpulla 2004]. NRF-1 plays thus, a relevant role in the integration of the nucleus-mitochondria interactions [Scarpulla 2002]. On the other hand, the nuclear respiratory factor 2 (NRF-2) also known as GA-binding protein or GABP, has also been implicated in mitochondrial gene regulation and mitochondrial biogenesis. NRF-2 is a multi-subunit nuclear transcription factor. It belongs to the E26 transformation-specific (Ets) family of proteins that binds the GGAA core DNA sequence. NRF-2 functions as a heterotetramer of two alpha subunits (NRF-2α) and two beta subunits (NRF-2β)—a unique property among Ets transcription factors. NRF-2α contains the Ets DNA binding domain, whereas NRF-2β contains the transactivating domain and the nuclear localizing signal. Both NRF-2 subunits are expressed widely throughout the body [Rosmarin et al. 2004]. GABPA is a unique gene in the human and murine genomes [Luo et al. 1999], and GABPα is the only protein that can recruit GABPβ to DNA to form the transcriptionally active complex. GABP regulates lineage-restricted myeloid and lymphoid genes that are required for innate immunity [Rosmarin et al. 2004], is required for cell cycle control in fibroblasts [Yang et al. 2007], and has been implicated in the regulation of more than one dozen mitochondrial genes [Ongwijitwat et al. 2005], being required for mitochondrial biogenesis [Yang et al. 2014]. Therefore, NRF-2 additionally works in concert with NRF-1 to integrate the nucleus-mitochondria transcription gene network and modulate mitochondrial biogenesis.

Coordinate expression of mitochondrial- and nuclear-encoded genes has been suggested to be mediated by hormone receptors within mitochondria stimulating the expression of mtDNA-encoded genes [Chen and Yager 2004]. Given that we have

previously reported the non-classical localization of AR in mitochondria of skeletal muscle cells from where the receptor could be mediating the antiapoptotic effects of testosterone, a goal in the present study was to elucidate one of the pathways that may contribute to the observed testosterone-induced mitochondrial preservation and protection, by the regulation of the expression of nuclear and mitochondrial genes that coordinately preserve the mitochondrial integrity and functionality. Thus, this effect of testosterone at mitochondrial level could be part of its protective action against H_2O_2-induced apoptosis in C2C12 cells. Here we tested the hypothesis that testosterone increases mitochondrial activity and mtDNA-encoded mRNA expression through AR-mediated up-regulation of *Nrf-1* gene transcription. We report that *Nrf-1* mRNA is up-regulated by testosterone, being this transcriptional effect mediated by AR in skeletal muscle cells. As consequence, NRF-1/TFAM/TFB2M/mitochondrial gene axis is activated, and testosterone ends up-regulating the mtDNA-encoded genes. In addition, the possibility that testosterone could directly influence mitochondrial oxidative phosphorylation gene transcription by way of cognate receptors present in mitochondria, has also been proposed. We computational tested the hypothesis of a direct regulation of mitochondrial genes by the AR. We reported several putative AR binding sequences in the mtDNA that could be mitochondrial androgen response elements, acting as enhancers of the mitochondrial encoded genes.

Materials and Methods

Materials

Testosterone was purchased from Sigma–Aldrich. Anti-NRF-1, anti-OPA1, and anti-TFB2M antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Alexa Fluor 488-conjugated anti-mouse secondary antibody was purchased from Thermo Fisher Scientific (Rockford, IL, USA). High Pure RNA Isolation kit (11828665001) was from Roche Diagnostics (Mannheim, Germany). High Capacity cDNA Reverse Transcription Kit (4368814) and SYBR® Select Master Mix were purchased from Applied Biosystems, and primer sets were from Invitrogen (Carlsbad, CA, USA). All other reagents used were of analytical grade.

Cell culture and treatment

C2C12 murine skeletal muscle cells, from the American Type Culture Collection (ATCC number: CRL-1772TM) at Manassas, VA 20108, were cultured in growth medium (Dulbecco's modified Eagle's medium) supplemented with 10% heat-inactivated (30 min, 56 °C) fetal bovine serum, 1% nystatin, and 2% streptomycin. Cells were incubated at 37 °C in a humid atmosphere of 5% CO_2 in air. Cultures were passaged every 2 days with fresh medium. Under these conditions, C2C12 myoblasts resemble the activated satellite cells that surround the mature myofibers and proliferate and

differentiate, participating in the repair of the tissue when a cellular injury exists [Yoshida et al. 1998]. Cells were cultured in chamber slides for microscopy or in 10 cm plates (Greiner Bio-One, Frickenhausen, Germany) for Western blots. The treatments were performed with 70-80% confluent cultures (120,000 cells/cm^2) in medium without serum for 30 min or during the longest treatment time that the experiment takes and this will be named Control condition. Then, treatments were carried out by adding 10^{-9} M testosterone at physiological concentration [Pronsato et al. 2010, 2012; Kerry et al. 2004] or the hormone vehicle, isopropanol (IPA) (the IPA percentage in the culture medium assay, of cells treated with the hormone or the hormone vehicle alone, was less than 0.001%) during the times indicated for each experiment. For AR evaluation, cells were treated with 10^{-8} M Flutamide for 60 min and then expose to 10^{-9} M testosterone during the time specified for each experiment. The induction of apoptosis with H_2O_2 was evaluated during the periods of time indicated for each experiment, by the dilution of H_2O_2 in culture medium without serum at a final concentration of 1 mM. To obtain a total cell lysate, a buffer composed of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.2 mM Na_2VO_4, 25 mM NaF, 1 mM PMSF, 20 µg/ml leupeptin, 20 µg/ml aprotinin and 20 µg/ml trypsin inhibitor, was employed. The lysates were collected by aspiration and centrifuged at 12,000 *g* during 5 min to separate the particulate fraction from the soluble fraction. Protein concentration from the supernatant was estimated by the method of Bradford [Bradford 1976], using bovine serum albumin (BSA) as standard.

Western blot analysis

Protein aliquots (25 mg) were combined with sample buffer (400 mM Tris-HCl (pH 6.8), 10% SDS, 50% glycerol, 500 mM dithiothreitol (DTT), and 2 mg/ml Bromophenol Blue, boiled for 5 min and resolved by 10% SDS-PAGE. Fractionated proteins were then electrophoretically transferred onto PVDF membranes (Immobilon-P; Millipore, Darmstadt, Germany), using a semi-dry system. Nonspecific sites were blocked with 5% nonfat dry milk in PBS containing 0.1% Tween-20 (PBS–T). Blots were incubated overnight with the appropriate dilution of the primary antibodies. The membranes were repeatedly washed with PBS–T prior incubation with horseradish peroxidase-conjugated secondary antibodies. The enhanced chemiluminescence (ECL) blot detection kit (Amersham, Buckinghamshire, England) was used as described by the manufacturer to visualize reactive products. For tubulin loading control, membranes were stripped with stripping buffer (62.5 mM Tris-HCl, pH 6.7; 2% SDS; 50 mM β-mercaptoethanol), washed with PBS 1% Tween-20 and then blocked for 1 h with 5% nonfat dry milk in PBS containing 0.1% Tween-20 (PBS–T). The blots were then incubated for 1 h with the antibody. After several washings with PBS-T, membranes were incubated with the secondary conjugated antibody. The corresponding immunoreactive bands were developed by means of ECL. Relative quantification of

Western blot signals was performed using ImageJ software (NIH, USA) [Abramoff et al. 2004].

Immunocytochemistry

After treatments, semi-confluent (60-70%) monolayers were washed with serum-free phenol red-free DMEM, and then fixed and permeabilized during 20 min at -20 °C with methanol to allow intracellular antigen labeling. After fixation, cells were rinsed 3 times with PBS. Non-specific sites were blocked for 30 min in PBS that contained 5% bovine serum albumin. Cells were then incubated overnight at 4 °C, in the presence or absence (negative control) of primary antibodies (1:50 dilution). The primary antibodies were recognized by fluorophore-conjugated secondary antibodies. Finally, the coverslips were analyzed by conventional fluorescence microscopy.

Quantitative Real Time RT-PCR

C2C12 cells were treated during different periods of time with 1 mM H_2O_2 or with 10^{-9} M testosterone or preincubated with Flutamide 10^{-8} M and then expose to 10^{-9} M testosterone for the times specified. After treatments, total RNA (10^{-6} cells/condition) was extracted using the High Pure RNA Isolation kit (Roche Diagnostics, Mannheim, Germany) and approximately 2 µg of total RNA was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., CA, USA) according to the manufacturer´s instructions. The RNA and cDNA purity has been checked by the measurement of 260/280 nm ratio. Quantitative measurement of real-time PCR was done using SYBR® Select Master Mix under the standard conditions recommended by the manufacturer. Primer sets to amplify murine cDNAs used in the analysis were as follows: glycelaldehyde 3-phosphate dehydrogenase (*Gapdh*) set: forward 5´CGTCCCGTAGACAAAATGGT3´, reverse 5´TTGATGGCAACAATCTCCAC3´; mitochondrial transcription factors A (*Tfam*) set: forward 5´CCCCTCGTCTATCAGTCTTGT3´, reverse 5´ CTGCTTCTGGTAGCTCCCTC 3´; nuclear respiratory factors 1 (*Nrf-1*) set: forward 5´ TCTCACCCTCCAAACCCAAC 3´, reverse 5´CCCGACCTGTGGAATACTTG3´; GA binding protein alpha (*Gabpa*) also named as nuclear respiratory factors 2 (*Nrf-2*) set: forward 5´ CCGGGGAACAGAACAGGAAA 3´, reverse 5´ ACGTTGTCCCCATTTTTGCG 3´; NADPH dehydrogenase subunit 1 (*ND1*) set: forward 5´CAACCATTTGCAGACGCCAT3´, reverse 5´TTGGGCTACGGCTCGTAAAG3´, NADPH dehydrogenase subunit 4 (*ND4*) set: forward 5´CTCCTCAGACCCCCTATCCA3´, reverse 5´AAATCCCTGCGTTTAGGCGT3´; cytochrome c oxidase subunit 1 (*Cox1*) set: forward 5´TGCAACCCTACACGGAGGTA3´, reverse 5´GTGGGCTTTTGCTCATGTGT3´; cytochrome c oxidase subunit 2 (*Cox2*) set: forward 5´TTGCTCTCCCCTCTCTACGC3´, reverse 5´TCTATTGGCAGAACGACTCGG3´; cytochrome b

(*CytB*) set: forward 5´GCCCTAGCAATCGTTCACCT3´, reverse 5´ATGGGGTGGGGTGTTTAGTG3´. The specificity of PCR products was confirmed by melting curve analysis. Relative quantification of gene expression was determined by the comparative C_T method [Giulietti et al. 2001; Livak and Schmittgen 2001].

AR motif analysis

Putative transcription factor binding sites (TFBS) of the AR were computational predicted and scanned within the murine mitochondrion genome utilizing a position-weight matrix (PWM) scan as implemented in PWMScan (https://ccg.epfl.ch/pwmtools/pwmscan.php) and described by Ambrosini and coworkers [Ambrosini et al. 2018]. Available mouse mitochondrion complete genome sequence (RefSeq: NC_005089.1) was extracted from NCBI Entrez gene database [Maglott et al. 2005]. PWMs were taken from the open access database JASPAR [Khan et al. 2018], HOCOMOCO [Kulakovskiy et al. 2018] and CIS-BP [Weirauch et al. 2014], which provide PWMs derived not only from SELEX experiments and individual promoter assays, but also from ChIP-Seq data using MEME for motif discovery [Bailey et al. 2009]. The P-value of a PWM score x is defined as the probability that a random k-mer sequence of the length of the PWM has a binding scorex given the base composition of the genome [Ambrosini et al. 2018]. A stringent p-value cut-off less than $1x10^{-5}$, that is recommended by developers of the PWMScan website [Ambrosini et al. 2018] has been employed. Only motifs with scores > 1000 for 10-15 bp sequences were selected. Each motif was lined to mouse mitochondrial genome by the employment of BLASTn with 100% homology.

Statistical analysis

Results are shown as means ± standard deviation (S.D.) Statistical differences among groups were determined by one-way analysis of variance (ANOVA) followed by the Fisher´s Least Significant Difference (LSD) test, a multiple comparison post hoc test. Differences with $p<0.05$ were considered to be statistically significant.

Results

Testosterone regulates nuclear and mitochondrial transcription factors gene expression in C2C12 muscle cells

NRF-1 and NRF-2 (also known as GA-binding protein or GABP), are primary transcription factors of nucleic DNA encoded mitochondrial proteins [Wu et al. 1999]. NRF-1 and NRF-2 in turn modulate the gene expression of mitochondrial transcription factors A (TFAM), B1 (TFB1M), and B2 (TFB2M), which are indispensable for the

activation of mitochondrial DNA transcription [Chen et al. 2009]. Thus, they play a key role in the integration of the transcription of nuclear- and mitochondrial-encoded genes. In order to evaluate if testosterone regulates the expression of the transcription factors that are responsible for the proper expression of the MRC proteins and thus, the correct mitochondrial functioning, the expression of *Nrf-1, Nrf-2* and *Tfam* genes were analyzed by quantitative PCR. Their relative levels were determined by the employment of the comparative C_T method described in *Methods*. C2C12 cell cultures were treated with 10^{-9} M testosterone during different periods of time, ranging between 30 min and 4 h, or pretreated with Flutamide 10^{-8} M for 60 min, and then expose to 10^{-9} M testosterone for the same times mentioned above. The mRNA was then isolated and the relative expression levels of the genes were quantified. The *Gapdh* was used as a reference gene.

In C2C12 cells, testosterone induced the upregulation of *Nrf-1* and *Nrf-2* genes, in a time dependent way, with a maximum expression at 30 min of testosterone treatment. From then on, the expression of *Nrf-1* and *Nrf-2* decreased gradually, reaching control levels after 4 h of treatment. Of relevance, the employment of the antagonist of AR, Flutamide, reduced testosterone effect, suggesting the participation of the AR in the positive modulation of these transcription factors (Figure 1). Similar results were obtained when the expression of NRF-1 protein was analyzed by Western blot assay and immunocytochemistry. C2C12 cultures were incubated with the hormone 10^{-9} M during the periods of time specified. In order to evaluate if the AR was mediating the effect of the hormone in this event, cells were treated in addition with flutamide (10^{-8} M, 60 min) prior to testosterone. Then, immunocytochemical detection of NRF-1 was performed on C2C12 cultures by fluorescence microscopy, or total homogenates were obtained and analyzed by Western blot assay. Testosterone was able to enhance NRF-1 expression over control in a time-dependent way, with a maximum of expression at 1h 30min. From then on, NRF-1 slightly decays. Pretreatment with Flutamide, almost completely blocks testosterone effect (Figure 2A). The employment of conventional microscopy and immunocytochemistry allowed us to observe besides, the nuclear localization of the transcription factor after testosterone treatment, where it exerts its function at transcriptional level. This effect was reduced by Flutamide (Figure 2B), that leads to NRF-1 permanence in cytosol and to clean nucleus.

Testosterone upregulated *Tfam* gene expression as well, after 4 h treatment, effect that was reduced by Flutamide pretreatment (Figure 1).

Testosterone induces the TFB2M expression that oxidative stress downregulates

As it was mentioned, TFAM is essential for the activation of mtDNA transcription, and TFB1M and TFB2M, acting as auxiliary factors for promoter recognition, are

necessary for basal transcription of mtDNA. With the aim of study the effect of the apoptotic agent on the transcription factor TFB2M in C2C12 cells, cultures were exposed to 1 mM H_2O_2 during different periods of time ranging from minutes to hours (15 and 30 min, 1, 2, 3, 4 and 6 h). Using a specific antibody against TFB2M, Western blot assay showed that H_2O_2 significantly reduced TFB2M expression respect to the Control, in a time-dependent way from 30 min of treatment (Figure 3A). On the other hand, when cells were treated with testosterone 10^{-9} M during different times (10 and 30 min, 2, 4 and 20 h), TFB2M enhanced its expression over control levels (cells treated with the vehicle of the hormone) from 30 minutes of testosterone treatment (Figure 3B). These results show a protective effect against H_2O_2-induced apoptosis that the hormone could be exerting by preserving the correct transcription of mtDNA.

H_2O_2-induced oxidative stress downregulates mitochondrial genes that encode MRC proteins in C2C12 muscle cells

MRC is one of the most important structural and functional parts of mitochondria. It consists of a series complexes of metalloproteins bound to the mitochondrial inner membrane that cooperate in electron transfer and proton pumping across the inner mitochondrial membrane. The better-known function of MRC is its ability to produce the majority (more than 90%) of cellular energy as ATP form, generating a large quantity of ROS as well. ROS play a significant role in redox regulation of gene expression, the control of cell proliferation and apoptosis, as well as serving as second messenger involved in the modulation many physiological and pathological activities. ROS also have the potential of causing oxidative damage to DNA and other critical molecules within the mitochondria. Mitochondrial dysfunction, such as reductions in protein and mRNA levels of the mitochondrial respiratory chain, is believed to be involved in aging and age-related skeletal muscle diseases, such as sarcopenia [Boengler et al. 2017; Peterson et al. 2012].

In order to evaluate the mitochondrial gene expression during H_2O_2-induced apoptosis in C2C12 cells, we analyzed by quantitative PCR, the relative levels of some mitochondrial DNA encoded proteins of the MRC, such as ND1 and ND4, cytochrome b and COX1 and COX2, which are components of complexes I, III, and IV, respectively. For this purpose, we employed the comparative C_T method described in the *Materials and Methods* section. Total RNA from cell cultures exposed to 1 mM H_2O_2 during different times, ranging between 30 min and 4 h, was isolated and employed to determine the relative levels of mRNA expression. The mRNA was then isolated and the relative expression levels were quantified. The *Gapdh* gene was used as a reference.

In C2C12 cells, H_2O_2 provoked the downregulation of *Cox1, Cox2, ND1, ND4* and *CytB* gene expression, reaching their lower levels after 3-4 h of treatment. These

results suggest that oxidative stress induced by H_2O_2 contributes to mitochondrial dysfunction by reducing the proteins that conform the MRC and thus altering the most important structural and functional parts of mitochondria (Figure 4).

Testosterone upregulates mitochondrial genes that encode MRC proteins in C2C12 muscle cells

NRF-1 stimulates transcription of the mitochondrial transcription factor TFAM by binding to an *Nrf-1* response element in the *Tfam* promoter [Virbasius et al. 1994]. Consequently, TFAM increases the transcription of mtDNA-encoded gene targets [Scarpulla 2002]. Given our previous results showing testosterone-induced increase of *Nrf-1* and *Tfam* transcription factors, we investigate then if it leads to the induction of the downstream stimulation of the transcription mitochondrial-encoded *Tfam* target genes. Thus, the expression of the mitochondrial-encoded mRNAs, *Cox1, Cox2, ND1, ND4* and *CytB* were analyzed by quantitative PCR using the comparative C_T method described in *Methods*. Cell cultures were treated with 10^{-9} M testosterone during different periods of time ranging between 30 min and 4 h, or pretreated with Flutamide 10^{-8} M for 60 min, and then expose to 10^{-9} M testosterone for the same times mentioned above. The mRNA was then isolated and the relative expression levels of the genes were quantified. *Gapdh* gene was used as a reference.

In C2C12 cells, testosterone induced the upregulation of the mitochondrial genes that encode the MRC proteins, in a time dependent way, with a peak of expression at 4 h of testosterone treatment for *Cox1, Cox2, ND4* and *CytB*, and a maximum expression from 1h to 4h of hormone treatment for *ND1*. Of relevance, pretreatment with Flutamide, almost totally abolish testosterone effect, pointing to the participation of the AR in the positive modulation of these mitochondrial genes (Figure 5). These results strongly suggest that testosterone indirectly regulates mitochondrial gene expression in the skeletal muscle, probably via the NRF/TFAM/TFBM-mitochondrial genes axis.

Testosterone promotes mitochondria preservation by the induction of the expression of OPA1 in C2C12 cells

OPA1 (optic atrophy protein 1) is a nuclear-encoded mitochondrial protein with similarity to dynamin-related GTPases, that helps to regulate mitochondrial stability and energy output. It is a widely expressed protein localized in the inner mitochondrial membrane, where it forms a diffusion barrier for proteins stored in mitochondrial cristae. It regulates mitochondrial fusion and cristae morphology and protects against apoptosis [Frezza et al. 2006; Olichon et al. 2003; Griparic et al. 2004]. Intrinsic

apoptotic signals mediated proteolytic processing leads OPA1 oligomers disassembly and caspase activator cytochrome C release into mitochondrial intermembrane spaces.

In order to evaluate the effect of testosterone on this key mitochondrial protein, C2C12 cells were treated with testosterone 10^{-9} M during different times (30 min, 1, 2, 4 and 6 h) to compare them to those treated with the vehicle of the hormone (IPA 0.001%) or maintained in DMEM (C). Western blot assay employing a specific antibody against OPA1 revealed that testosterone was able to increase OPA1 expression in a time dependent way, being its highest level after 6h treatment (Figure 6). This result supports the idea of the protective effect of testosterone against apoptosis at mitochondrial level, in skeletal muscle.

Prediction of the putative transcription factor binding sites (TFBSs) of AR in murine mitochondrial genome

Knowing where transcription factors (TFs) bind to the genome is the key to understanding gene regulation [Narlikar and Ovcharenko 2009]. The prediction of TFBSs is a big challenge for computational biologists, mainly as increasing amounts of sequence data become available. Prediction of TFBSs is usually performed by scanning a DNA sequence of interest with a position weight matrix (PWM) for a TF of interest [Hannenhalli 2008; Garcia-Alcalde et al. 2010] and many pattern-matching tools have been developed for this purpose. A PWM comprises weights for each base at each motif position. By summing up weights at corresponding positions, a binding score can be computed for any base sequence of the same length as the PWM. Thus, PWMs became an essential computational tool and model of choice to describe sequence binding specificities of particular TF-DNA interactions. Large collections of TF specificity matrices are currently available from public libraries such as JASPAR [Khan et al. 2018], CIS-BP [Weirauch et al. 2014], or HOCOMOCO [Kulakovskiy et al. 2018].

In order to identify if the mouse mitochondrial genome contains sequence-specific binding sites for the AR, from which it could be mediating a direct transcriptional regulation of mitochondrial genes, we performed an in silico genome analysis. The PWMs of AR were used to scan all the mouse mitochondrial genome, obtained in FASTA format from NCBI (RefSeq: NC_005089), for putative AR-binding sites, by the employment of the PWMScan tools [Ambrosini et al. 2018]. The search led to the identification of several putative TFBSs with *p*-value < 0.0001 and matches scores over 1000, located at various sites of the mitochondrial genome (Table 1). Two sequences located in the regulatory site of the genome, the D-loop region, and the others within the ribosomal subunit genes 12S RNA and 16S RNA, the tRNA-Leu and tRNA-Val and within the structural genes *ND1* and *Cox1* (Figure 7). Besides, some putative TFBSs have been also detected in the complementary light chain (Table 1), one of them within ND6 gene. The AR matrix obtained from JASPAR do not reveal any

specific AR binding sequence with cut off score 1000. Of relevance, all putative TFBSs obtained were aligned with mouse mitochondrion complete genome by the employment of BLASTn, obtaining 100% homology between the queries and a site in the genome (data not shown), confirming the presence of these sequence-specific binding sites for the AR in mtDNA. These results show the presence of sequence inside the mitochondrial genome, that the AR could recognize, bind and exert its function as transcription factor by modulating mitochondrial gene transcription.

Discussion

Numerous *in vitro* and *in vivo* studies demonstrate that the androgen-AR signaling pathway is necessary for skeletal muscle development and for sustaining muscle mass, strength and protein synthesis [Herbst et al. 2004]. We have formerly reported that testosterone protects skeletal muscle C2C12 cells against apoptosis through a mechanism involving intermediates of the apoptotic intrinsic pathway and the AR [Pronsato et al. 2012]. It is well known that mitochondria play a key role in apoptosis triggered by many stimuli [Herynk et al. 2004]. Of significance to our work, the presence of steroid hormone receptors in this organelle has been demonstrated in a range of cells [Monje et al. 2001; Cammarata et al. 2004; Chen et al. 2004; Yang et al. 2004; Milanesi et al. 2008, 2009; Solakidi et al. 2005]. Indeed, our previous work, have demonstrated the presence of functional androgen binding sites in mitochondria [Pronsato et al. 2013]. The biochemical and immunological similarity between the reactive entities detected in mitochondria with the AR allow us to propose the presence of extranuclear AR in the C2C12 murine myoblast cell line. In this work, we demonstrate that testosterone through its receptor regulates mitochondrial respiratory chain biogenesis via the NRF/TFAM/TFB2M-mitochondria axis, in skeletal muscle cells. Testosterone prevents mitochondrial dysfunction strengthen thus, its antiapoptotic effect at mitochondrial level, probably directly mediating its function the non-classical mitochondrial AR.

With advancing age, mitochondria are often reported to diminish in volume and function within skeletal muscle. The mechanisms responsible for age-related mitochondrial loss are not fully understood and continue to be elucidated. To produce fully functioning, high-quality organelles, coordination between the nuclear and mitochondrial genomes is necessary to produce protein products in the correct stoichiometry. The biogenesis of mitochondria needs the expression of several genes encoded by both nuclear and mitochondrial genomes [Garesse and Vallejo 2001]. However, since the protein coding capacity of mtDNA is limited to 13 respiratory subunits, nuclear genes must provide the vast majority of products required for mitochondrial oxidative functions and biosynthetic capacity. Additionally, nuclear genes must play a major role in controlling mitochondrial transcription, translation,

and DNA replication. The NRF transcription factor has been suggested to play a key role in the integration of the transcription of nuclear- and mitochondrial-encoded genes [Evans 1989]. It is activated by the peroxisome proliferator activated receptor gamma coactivator 1 alpha (PGC-1α), the master regulator of mitochondrial biogenesis. NRF-1 and NRF-2, act on the majority of nuclear genes encoding subunits of the respiratory complexes. They are also involved in the expression of mitochondrial transcription and replication factors, such as TFAM, involved in the regulation of the transcription and replication of the mitochondrial genome [Clayton 1992, Dairaghi et al. 1995, Puigserver and Spiegelman 2003], heme biosynthetic enzymes, and other proteins essential for respiratory function. TFB1M and TFB2M are also subject to NRF-1 and NRF-2 regulation, suggesting that these factors could be involved in the integration of the expression of respiratory subunits with components of the mitochondrial transcriptional machinery [Virbasius and Scarpulla 1994]. Regulation of these transcription factors and thus, the modulation of the mitochondrial genome by estrogens, have been well reported, being its receptor, a key modulator of these events [Chen et al. 2009; Mattingly et al. 2008]. However, little is known about the effects of androgens on the regulation of the expression of these factors and the proteins regulated by them. In this work, we demonstrate that testosterone at physiological concentration induce the expression of *Nrf-1* and *Nrf-2*, effect that leads to an increased expression of not only *Tfam*, but also, TFB2M, in skeletal muscle cells. Of relevance, these events are reverted in the presence of the AR antagonist, Flutamide. Because testosterone induces the expression of *Tfam*, and possibly TFB2M via activation of NRF-1 and NRF-2, it is likely that T and AR stimulate the mtDNA transcription via induction of these mitochondrial transcription factors expressions. Accordingly, considering that TFAM and TFBs transcribe the mitochondrial genome, testosterone treatment resulted in an increase of mRNA transcription levels of mtDNA-encoded protein subunits that comprising the mitochondrial respiratory chain. mRNA expression of the subunits of complex I, III and IV of MRC, *ND1, ND4, CytB*, *Cox1* and *Cox2* were increased after testosterone treatment. The employment of Flutamide was able to almost totally inhibit the effect of the hormone, showing the requirement of the AR for the modulation of these effects. These findings suggest that testosterone exerts a relevant role in the modulation of mitochondrial gene expression in skeletal muscle. Consistent with our observations in skeletal muscle, others reported an important role of AR in maintaining oocyte quality and fertility by controlling the signals of PGC1α-mediated mitochondrial biogenesis in granulosa cells [Wang et al. 2015]. Testosterone deficiency in brain causes an important reduction in mtDNA gene expression namely *ND1*, *ND4*, cytochrome b, and *Cox1* and *Cox3*, in the hippocampus, as a consequence of the downregulation of PGC-1α, its downstream transcriptional factors, NRF-1 and NRF-2, and mitochondrial transcription factors A and B2, assigning androgen an important role in mitochondrial hippocampus [Hioki et al. 2014]. With regard to skeletal muscle, testosterone administration in addition to low-intensity

physical training in mice has been associated with improvements in muscle mass, grip strength, spontaneous movements, and respiratory activity, being these improvements related to increased muscle mitochondrial biogenesis and quality [Guo et al. 2012]. Usui et al. firstly reported that testosterone-treated mice exhibited elevated heat production, associated to the activation of PGC-1α/NRF/TFAM/mitochondrial genes axis, deriving the increase in energy expenditure induced by testosterone, from elevated mitochondrial biogenesis in skeletal muscle [Usui et al. 2014]. Our results deepen the knowledge about the role of testosterone and its receptor in mitochondrial biogenesis as a key action of their protective effect against H_2O_2-induced apoptosis in C2C12 skeletal muscle cells.

Accompanied by mitochondrial biogenesis, mitochondrial dynamics (i.e. fission and fusion processes) is vital for accurate mitochondrial function, and even minor failure in mitochondrial dynamics are related to muscle pathologies such as sarcopenia [Gorgey et al. 2019; Huang et al. 2019]. Mitochondria in skeletal muscle undergo fusion and fission in response to the state of the muscle fiber to guarantee there is enough capacity for energy production during demanding events. The mitochondria undergo fusion in skeletal muscle to generate a tightly regulated reticulum that rises the efficiency of oxidative phosphorylation [Glancy et al. 2015]. Mitochondrial fusion is regulated by the outer membrane fusion proteins mitofusion 1 and 2 (MFN 1–2) and inner membrane fusion protein optic atrophy 1 (OPA1). Decreases in these proteins are related to mitochondrial dysfunction, induction of apoptosis and muscle atrophy [Chen et al. 2010; Olichon et al. 2003]. We found that testosterone, which enhances key intermediates factors of mitochondrial biogenesis, also stimulates mitochondrial fusion by upregulating OPA1 expression. Thus, testosterone helps to maintain balanced mitochondrial dynamics in skeletal muscle, ensuring a proper mitochondrial and, therefore, cell function.

H_2O_2 has been demonstrated to be a signaling molecule implicated in many cellular functions such as apoptosis [Singh & Singh 2008; Vasconsuelo et al. 2008], differentiation [Steinbeck et al. 1998; Orzechowski et al. 2002], and proliferation [Sigaud et al. 2005]. Cell exposure to exogenous H_2O_2 has been used to investigate multiple signaling pathways related to smooth muscle contraction [Jin and Rhoades 1997], proliferation [Baas and Berk 1995] and apoptosis [Li et al. 1997]. H_2O_2 induces apoptosis in C2C12 cells in a time-dependent manner. At short times of exposure, H_2O_2 induces the activation of a cell defense response to avoid apoptosis, while at long treatment times, the programmed cell death finally starts. The loss of the mitochondrial membrane potential, the mitochondrial permeability transition pore (mPTP) opening and cytochrome c release take place, and it is at this time when the protective action of testosterone against apoptosis is evidenced [Pronsato et al. 2013, 2016]. In this research it has been demonstrated that H_2O_2-induced oxidative stress significantly downregulates the mRNA expression of the MRC proteins *ND1, ND4, CytB,*

Cox1 and *Cox2* respect to the Control in C2C12 cells. In addition, the auxiliary factor for promoter recognition, TFB2M was also reduced by the exposure to the apoptotic agent in a time dependent manner. Thus, it is probably that by the inhibition of NRF/TFAM/TFB2M/mitochondrial genes axis, the apoptotic agent destabilize mitochondrial integrity and functionality, leading finally to apoptosis of skeletal muscle cells. These effects were totally opposite to those obtained with physiological concentrations of testosterone treatment; strengthen the antiapoptotic role of the steroid in skeletal muscle.

One prevailing and indirectly view of hormonal regulation of mitochondrial transcription is that the hormonal stimulus primarily leads to the induction of the activation of nuclear-encoded mitochondrial transcription factors, which secondarily stimulate mitochondrial RNA synthesis, after translocating to the organelle [Pillar and Seitz 1997]. Alternatively, transcription factors can be imported into mitochondria and alter transcription from the mitochondrial genome as 'direct regulators' of mitochondrial gene expression. The identification of glucocorticoid [Demonacos et al. 1993; Scheller et al. 2000; Moutsatsou et al. 2001] estrogen [Monje et al. 2001; Cammarata et al. 2004; Chen et al. 2004; Yang et al. 2004; Milanesi et al. 2008, 2009] and thyroid [Wrutniak et al. 1995; Casas et al. 2003] hormone receptors in mitochondria and the presence, in the mitochondrial genome, of nucleotide sequences not only with high homology to hormone-responsive elements (HRE), but also with high capability to bind steroid and thyroid receptors, strengthen the notion of a direct effect of these hormones on mitochondrial transcription [Sekeris 1990; Demonacos et al. 1996; Wrutniak et al. 1995; Casas et al. 1999; Enriquez et al. 1999; Chen et al. 2004; Psarra et al. 2006]. However, lesser is known about the androgen receptor. Although we have previously demonstrated the mitochondrial localization of the AR capable of selectively binding androgens [Pronsato et al. 2013], a critical question remains unknown about the functional role of the mtAR in the global antiapoptotic effects of testosterone in skeletal muscle. Is the localization of AR in mitochondria a mechanism by which the cell synchronizes the nuclear and mitochondrial transcriptional responses for integrated responses to androgen? Or, do the effects of testosterone on mitochondria have particular roles in modulating transcription of certain mitochondrial genes?

To begin to elucidate this issue, we employed computational tools to look for specific DNA sequence, in mouse mitochondrial genome, that could act as potential androgen response elements (ARA) from where AR could modulate mitochondrial gene expression. Unlike nuclear genes, that often has multiple promoters, all mitochondrial genes are expressed together from only three promoters encoded in the regulatory D-loop region [Montoya et al. 1982], which are recognized by the mitochondrial basal transcriptional machinery: the mitochondrial RNA polymerase (Polrmt), and the mitochondrial transcription factors TFAM and TFB2M [Falkenberg et

al. 2007; Cotney et al. 2009]. The resulting three polycistronic transcripts do not undergo splicing (because of the absence of introns in the mtDNA), but are processed by an RNAse that remove tRNAs to release the mRNA and rRNA [Ojala et al. 1981] before mRNA translation in the mitochondrial matrix. In this work we have identify several putative AR binding sites in murine mitochondrial genome, that are present not only at the regulatory region (D-loop) of the mtDNA, where the promoters are located, but also at other sites (within structural genes and at the ribosomal RNA or tRNA genes). The presence of TFBSs also in sites other than the D-loop suggests possible effects not only in the initiation but also at other steps of the mitochondrial transcription process, such as transcript elongation, release and processing.

As a conclusion, the presence of common nucleotide sequences in the nuclear and mitochondrial genomes to which AR could bind, suggests the possibilities of a coordination of transcription between the two genomes. Thus, testosterone could, by a direct AR–DNA interaction stimulate parallel transcription in both nucleus and mitochondria. Additionally, by the induction of nuclear-encoded mitochondrial transcription factors, the hormone could produce the same effect. Thus, AR could directly regulate mitochondrial transcription or indirectly lead to the same effect by activating NRFs/TFAM-TFB2M/mitochondrial genes axis. Our study highlights the key role of androgens for mitochondrial gene expression in skeletal muscle and provides an explanation of the antiapoptotic effect of the hormone in aged-skeletal muscle underlying age-related reduction in mitochondrial proteins, function and quality. These data in addition to our previous findings sustain the significant role of testosterone in the inhibition of several cellular pathways in muscle cells that acting in concert conduce to apoptosis. Clearly, additional studies are necessary to further characterize the relative role of the muscle cell mtAR in mitochondrial biogenesis and to clarify the signaling mechanisms that mediate the antiapoptotic action of testosterone through the apoptotic intrinsic pathway in skeletal muscle cells, and its relationship with myopathies associated with hormonal dysregulation.

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Figure legends

Figure 1: Testosterone upregulates *Nrf* and *Tfam* transcription factors gene expression in C2C12 cells. Transcript levels of *Nrf-1, Nrf-2* and *Tfam* were determined by Real Time PCR as described in *Methods*, using C2C12 cultures preincubated with 10^{-8} M

Flutamide for 60 min and then exposed to 10^{-9} M Testosterone for 30 min (T + F 30), 1 h (T + F 1 h), 3 h (T + F 3 h) and 4 h (T + F 4 h) or treated with 10^{-9} M Testosterone alone for the same periods of time; (C) Untreated cells. Transcript levels are expressed in arbitrary units related to the expression of *Gapdh* gene expression. Averages ± S.D. are given; * $p < 0.05$ respect to Control condition; # $p < 0.05$ respect to the counterpart T condition.

Figure 2: Testosterone induces NRF-1 protein expression and its nuclear location in C2C12 cells. **A)** C2C12 cultures preincubated with 10^{-8} M Flutamide for 60 min and then exposed to 10^{-9} M Testosterone for 30 min (T + F 30), 1 h 30min (T + F 1h 30´) and 3 h (T + F 3h) or treated with 10^{-9} M Testosterone alone for the same periods of time; (C) Untreated cells. Cell lysates were prepared and subjected to Western blot analysis using anti-NRF-1 antibody. β-Tubulin levels are shown as protein loading control. The bands were quantified by densitometry. **B)** Cells grown on coverslips were preincubated with 10^{-8} M Flutamide for 60 min and then exposed to 10^{-9} M Testosterone for 30 min (T + F 30), 1 h (T + F 1h) and 4 h (T + F 4h) or treated with 10^{-9} M Testosterone alone for the same periods of time; (C) Untreated cells. Then cells were labeled with anti-NRF-1 as described in *Methods*. At least ten fields per slide were examined by fluorescence microscopy. Representative photographs are shown. Magnification: 63X

Figure 3: Time course of H_2O_2 and testosterone modulation of TFB2M expression in muscle cells. **A)** C2C12 cells were incubated with 1 mM H_2O_2 for the indicated time periods or maintained in DMEM (C). Cell lysates were prepared and subjected to Western blot analysis using anti-TFB2M antibody. β-Tubulin levels are shown as protein loading control. The bands were quantified by densitometry. The blot is representative of three independent experiments with comparable results. Averages ± S.D are given. *$p < 0.05$ respect to the control (C). **B)** C2C12 cells were treated during the indicated periods of time with 10^{-9} M testosterone or IPA (0.001%). Western blot analyses were performed using anti-TFB2M antibody. β-Tubulin level is shown as loading control. The blots are representative of three independent experiments with comparable results. Densitometric quantification of the blot is shown. Averages ± S.D are given. *$p < 0.05$ respect to the counterpart IPA control condition.

Figure 4: H_2O_2-induced oxidative stress downregulates mitochondrial gene expression in C2C12 cells. Transcript levels of *ND1, ND4*, *Cox1*, *Cox2*, and *CytB* were determined by Real Time PCR as described in *Methods*, using C2C12 cultures exposed to 1 mM H_2O_2 for 30 min (H30), 1 h (H 1h), 3 h (H 3h) and 4 h (H 4h) or (C) untreated. mRNA levels are expressed in arbitrary units related to the expression of *Gapdh* gene. Averages ± S.D. are given; *$p < 0.05$ respect to Control condition.

Figure 5: Testosterone upregulates mitochondrial genes of the MRC in C2C12 cells. Transcript levels of *ND1, ND4, Cox1, Cox2*, and *CytB* were determined by Real Time PCR as described in *Methods*. C2C12 cultures were preincubated with 10^{-8} M Flutamide for 60 min and then exposed to 10^{-9} M Testosterone for 30 min (T + F 30), 1 h (T + F 1 h), 3 h (T + F 3 h) and 4 h (T + F 4 h) or treated with 10^{-9} M Testosterone alone for the same periods of time; (C) Untreated cells. Transcript levels are expressed in arbitrary units related to *Gapdh* gene expression. Averages ± S.D. are given; * $p < 0.05$ respect to Control condition; # $p < 0.05$ respect to the counterpart T condition.

Figure 6: Time course of testosterone modulation of OPA1 expression in muscle cells. C2C12 cells were treated during the indicated periods of time with 10^{-9} M testosterone, IPA (0.001%) or maintained in DMEM (C). Western blot analyses were performed using anti-OPA1 antibody. β-Tubulin level is shown as loading control. The blots are representative of three independent experiments with comparable results. Densitometric quantification of the blot is shown. Averages ± S.D are given. *$p < 0.05$ respect to the counterpart IPA control condition.

Figure 7: Prediction of putative TFBSs of AR in murine mitochondrial genome by PWMScan. A) PWMs Logo for AR-binding motif sequence obtained from CIS-BP (M1456_1.02 Ar) and HOCOMOCO (ANDR_MOUSE.H11MO.0.A and ANDR_MOUSE.H11MO.1.A) databases. B) Map of the *Mus Musculus* (NC_005089) mitochondrial genome. The mouse mitochondrial DNA (mtDNA) comprises 16,299 bp. mtDNA forms an inner light strand (L-strand) and an outer heavy strand (H-strand) that encodes different genes. The H-strand, encodes 28 genes: 2 rRNAs (rRNAs 12S y 16S), 14 tRNAs (tRNA -Phe, -Val, -Leu, -Ile, -Met, -Trp, -Asp, -Lys, Gly, -Arg, -His, -Leu, -Ser, -Thr) and 12 polypeptides (ND1, ND2, ND3, ND4, ND4L, ND5, COX1, COX2, COX3, ATP6, ATP8, CytB), while the L-strand, encodes only 8 tRNAs (tRNA -Gln, -Ala, -Asn, -Cys, -Tyr, -Ser, -Glu, -Pro) and a single polypeptide, ND6. Colors identify the different genes: rRNAs (orange), tRNAs (blue), proteins encoding subunits of the MRC complexes I (pink), III (light blue), IV (green), and V (red). Non-coding region, D-loop, is represented by color yellow. The gray call boxes contain the specific sequence binding sites predicted for the AR and their location in the genome: in red those located in H-strand, in blue those located in L-strand.

Table 1: Sequences showing putative TFBSs of AR detected in the mouse mitochondrial genome[a]

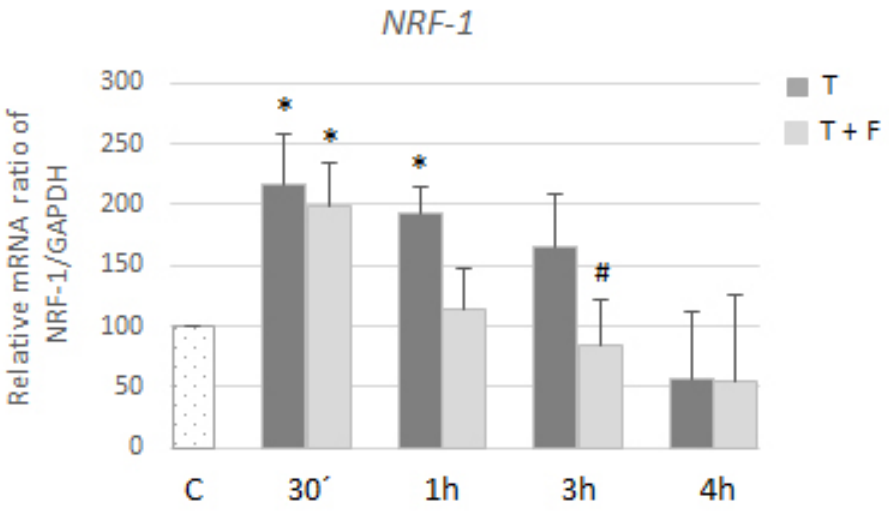
Consensus: brTGTTCytw

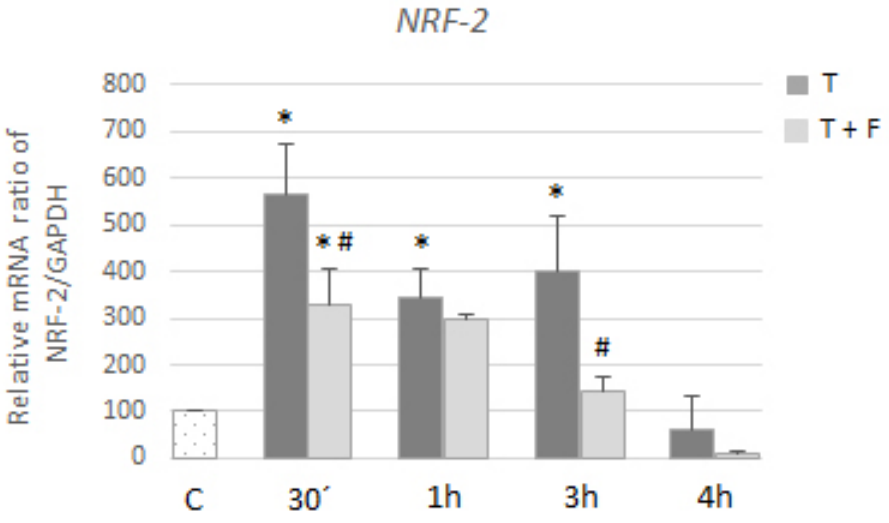
RGvACAbTvTGTbCYb

dbYbTGTbCYbd

Genome Position		Sequence	Strand H (+) L (-)	Score	p-Value	Location
619	631	AGCCTGTTCTAT	+	1070	1.05e-04	rRNA 12S
800	810	AATGTTCTTT	-	1209	7.04e-05	
1081	1097	TGAACACTCTGAACTA	+	1023	2.79e-05	tRNA-Val
1244	1256	TGCTTGTTCTTA	-	1087	9.18e-05	
2041	2051	CTTGTTCCTT	+	1028	2.27e-04	rRNA 16S
2714	2726	ACCTTGTTCCCA	+	1002	1.73e-04	tRNA-Leu
2749	2759	AGTGTTCTTT	+	1134	1.25e-04	ND1
2833	2843	TATGTACCCT	-	1021	2.48e-04	
3254	3266	TATGTGTTCTTG	-	1180	3.99e-05	
4154	4164	CATGTTCCTA	-	1400	5.27e-06	
5326	5336	TATGTTCATT	+	1139	1.13e-04	COX1
5692	5708	TGTTCATCCTGTTCCT	-	1182	1.19e-05	
6459	6471	ACTATGTTCTAT	+	1008	1.64e-04	COX1
9789	9801	ACTCTGTTCATT	-	1164	4.74e-05	
10123	10135	AATCTGTTCCGT	-	1080	9.70e-05	
13745	13757	TTTGTGTGCTTA	-	1030	1.40e-04	ND6
15961	15973	ACTGTGTGCTGT	-	1230	2.38e-05	D-loop
16239	16249	AATGTTCCGT	+	1013	2.68e-04	D-loop

a. The numbers refer to the position of the sequences in the genome. Denoted also are the genes in which the putative TFBSs sequences have been detected.


NRF-1
Relative mRNA ratio of NRF-1/GAPDH
300
250
200
150
100
50
0
T
T + F
*
*
*
#
C
30´
1h
3h
4h


NRF-2
Relative mRNA ratio of NRF-2/GAPDH
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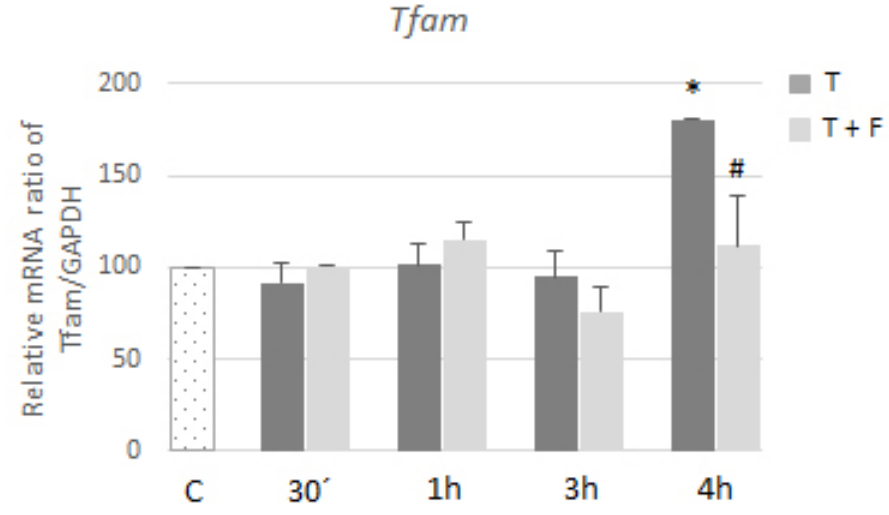
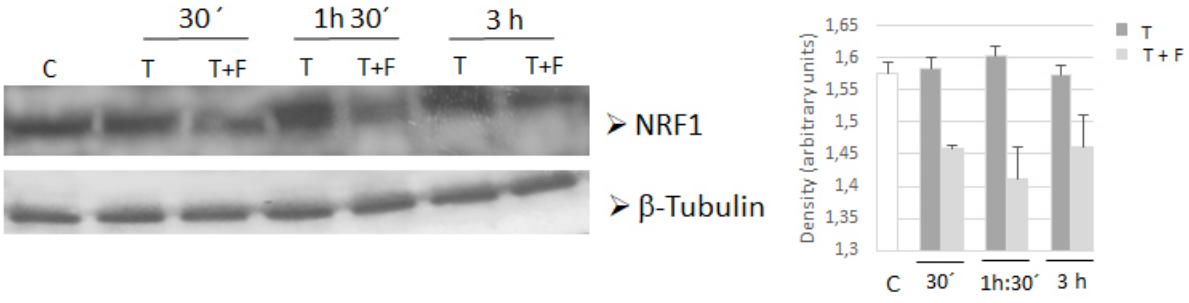
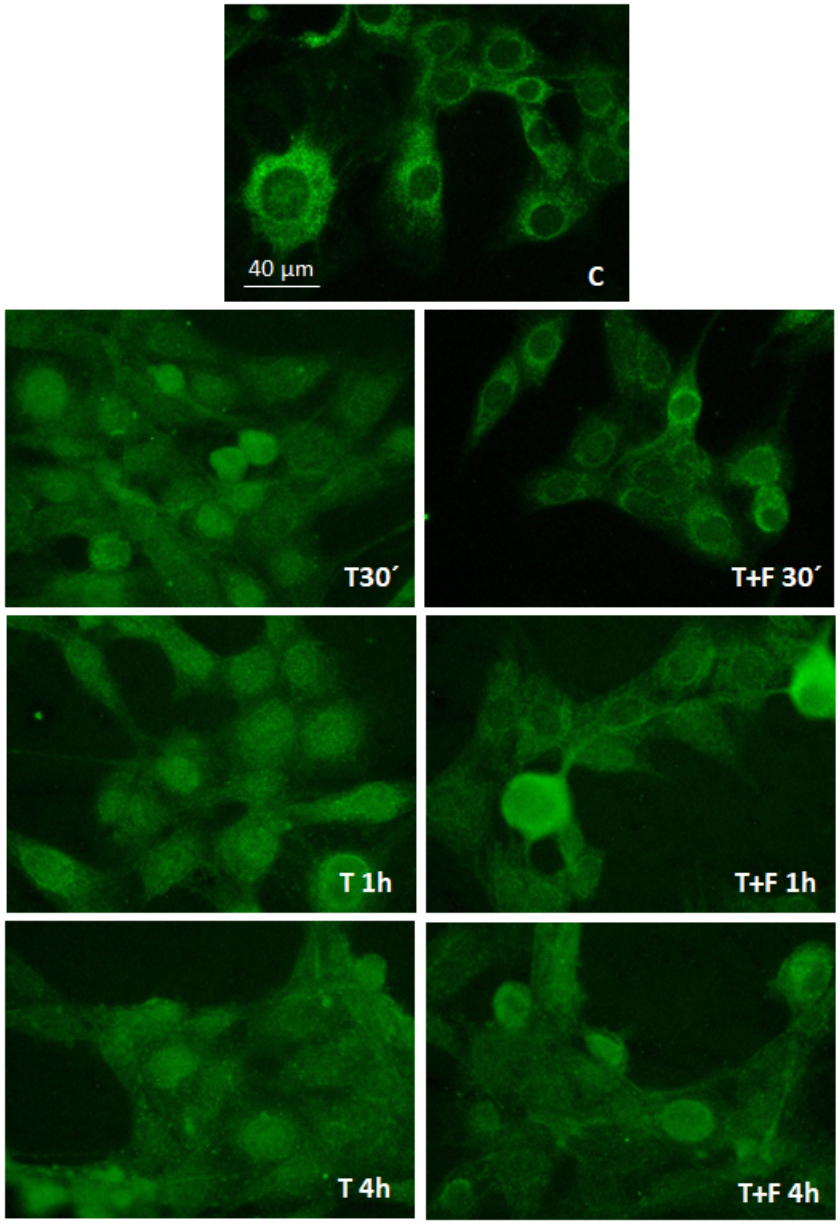
700
600
500
400
300
200
100
0
T
T + F
*
*#
*
*
#
C
30´
1h
3h
4h

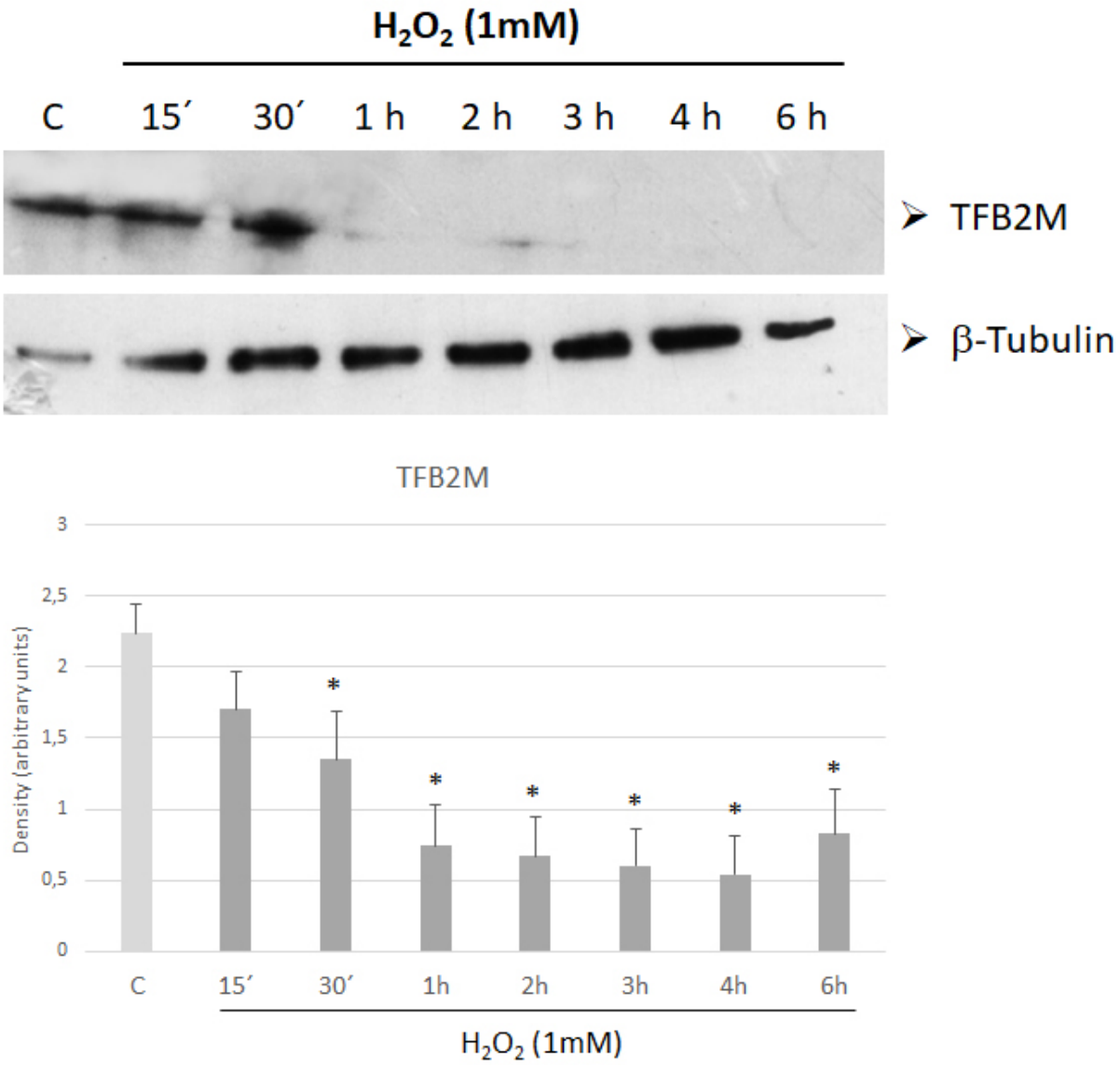

Tfam
Relative mRNA ratio of Tfam/GAPDH
200
150
100
50
0
T
T + F
*
#
C
30´
1h
3h
4h

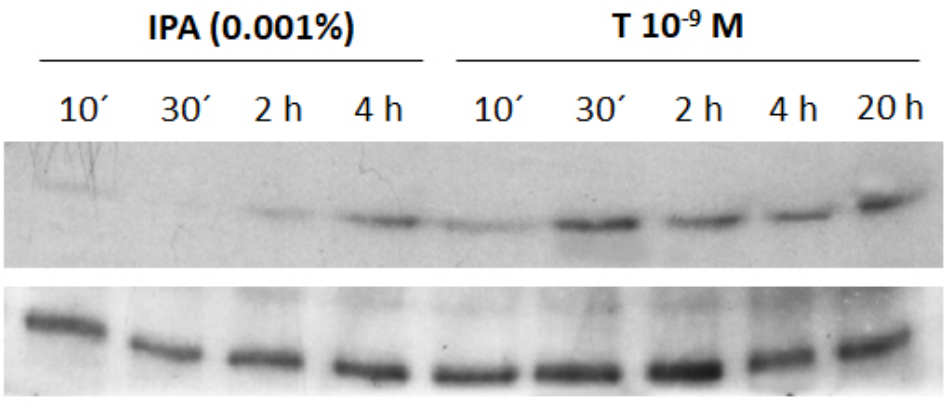
A.

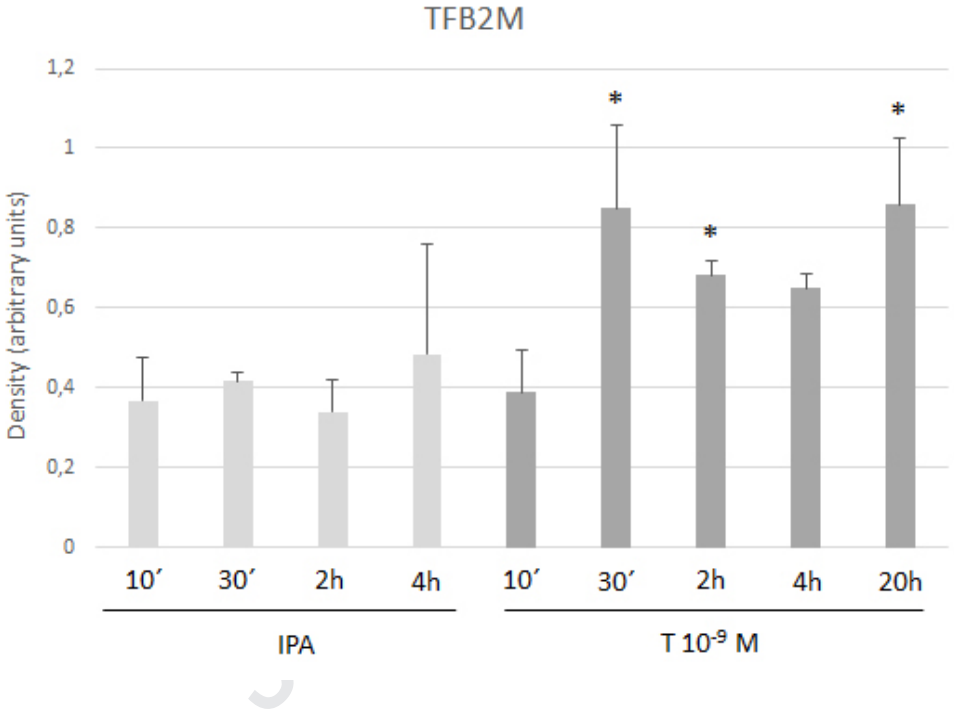

30´
1h 30´
3 h
C
T
T+F
T
T+F
T
T+F
➢ NRF1
➢ β-Tubulin
NRF1
Density (arbitrary units)
1,65
1,6
1,55
1,5
1,45
1,4
1,35
1,3
C
30´
1h:30´
3 h
T
T + F

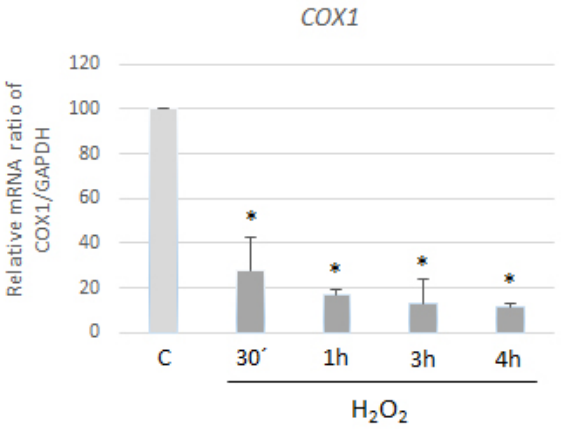
B.


40 µm
C
T30´
T+F 30´
T 1h
T+F 1h
T 4h
T+F 4h


H2O2 (1mM)
C
15´
30´
1 h
2 h
3 h
4 h
6 h
TFB2M
β-Tubulin
TFB2M
Density (arbitrary units)
3
2,5
2
1,5
1
0,5
0
C
15´
30´
1h
2h
3h
4h
6h
H2O2 (1mM)


IPA (0.001%)
T 10-9 M
10´ 30´ 2 h 4 h 10´ 30´ 2 h 4 h 20 h
TFB2M
β-Tubulin


TFB2M
Density (arbitrary units)
1,2
1
0,8
0,6
0,4
0,2
0
*
*
*
10´ 30´ 2h 4h 10´ 30´ 2h 4h 20h
IPA
T 10-9 M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Relative mRNA ratio of COX1/GAPDH
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100
80
60
40
20
0
C
30´
1h
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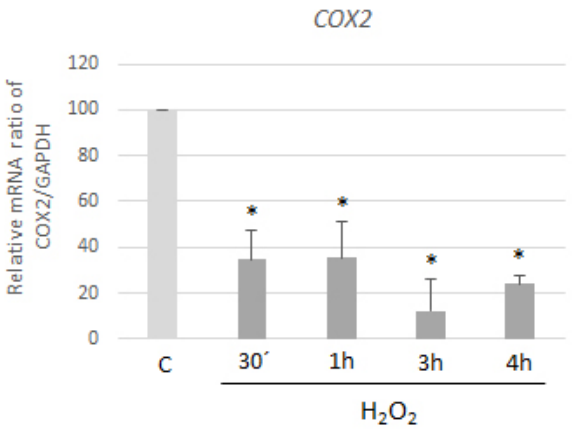
H2O2


COX2
Relative mRNA ratio of COX2/GAPDH
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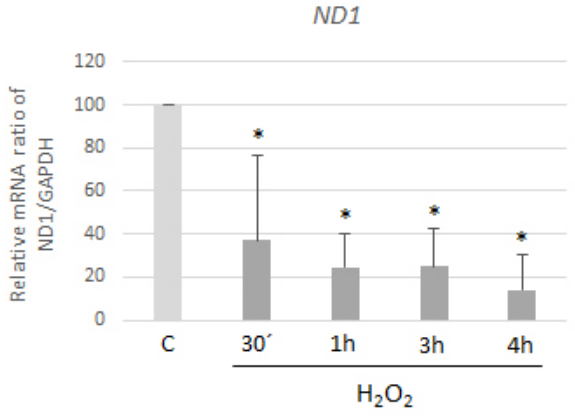
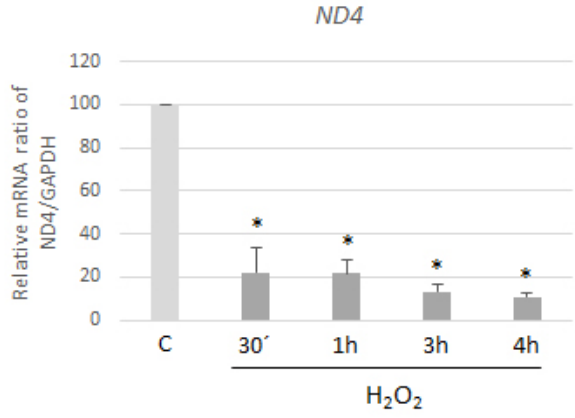
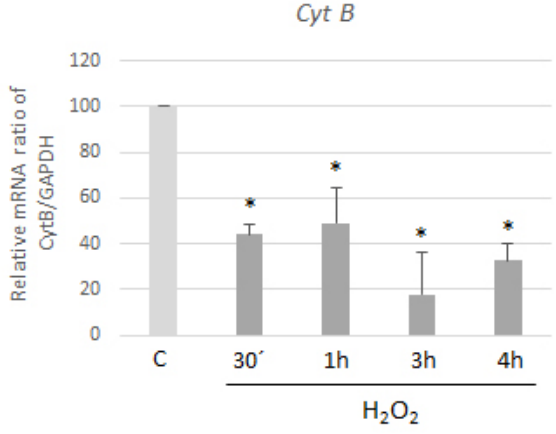
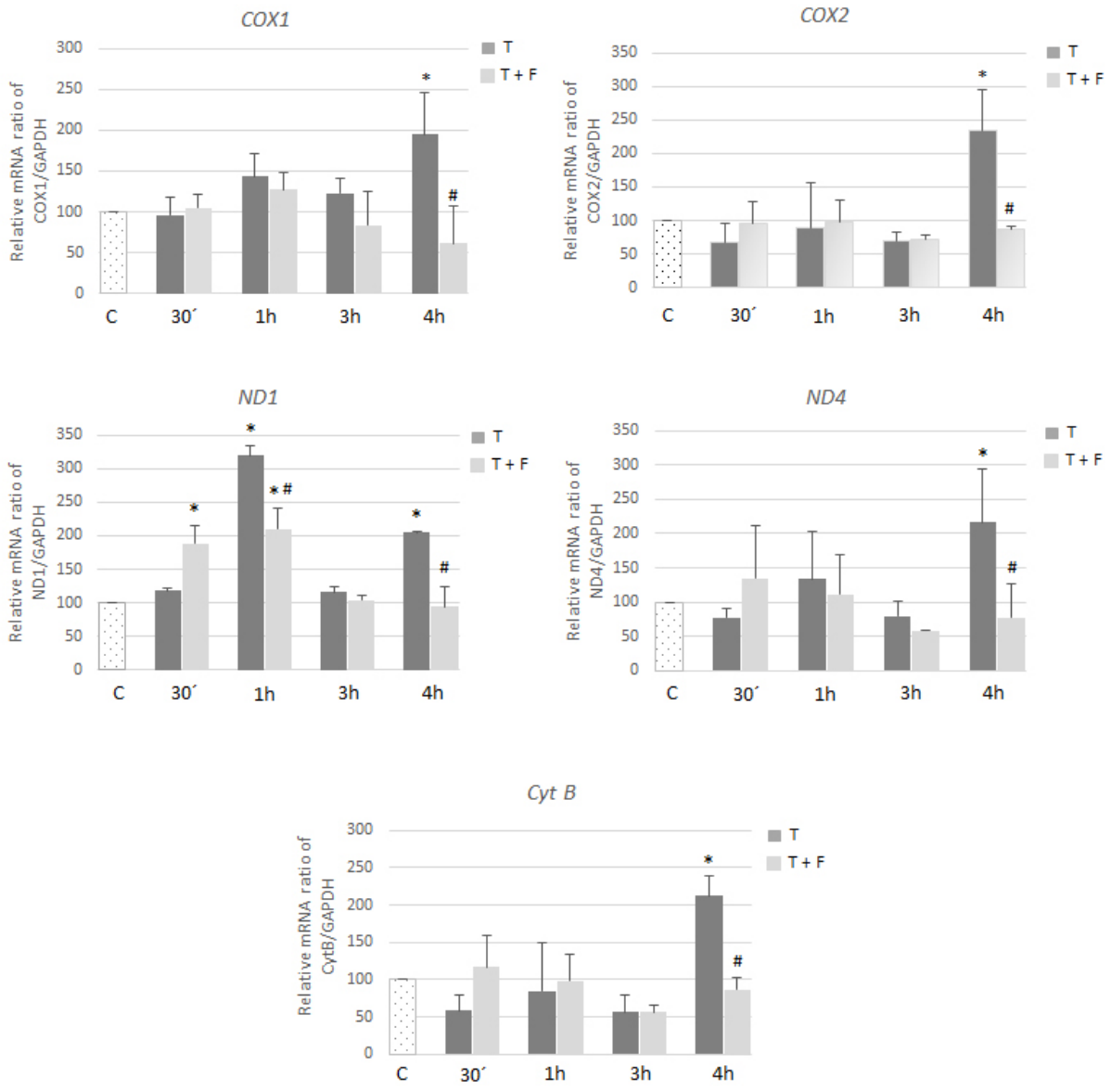
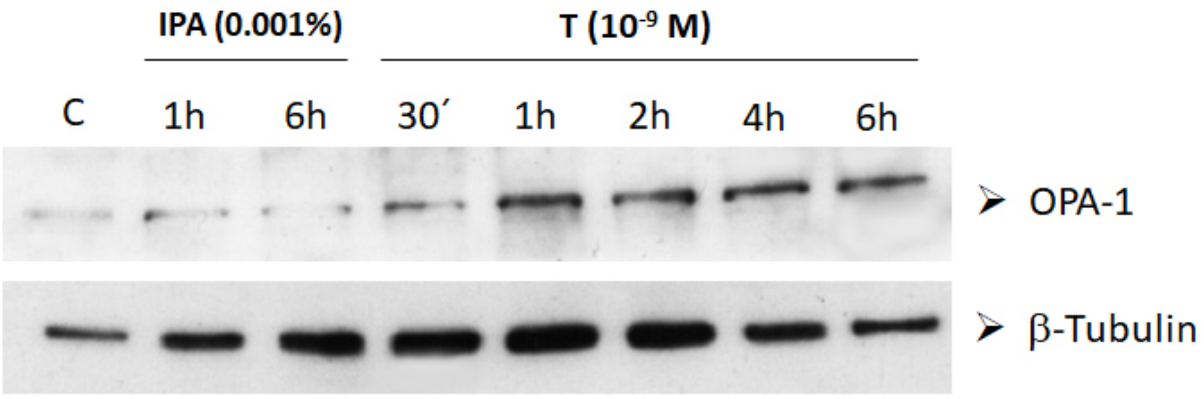
100
80
60
40
20
0
C
30´
1h
3h
4h
H2O2

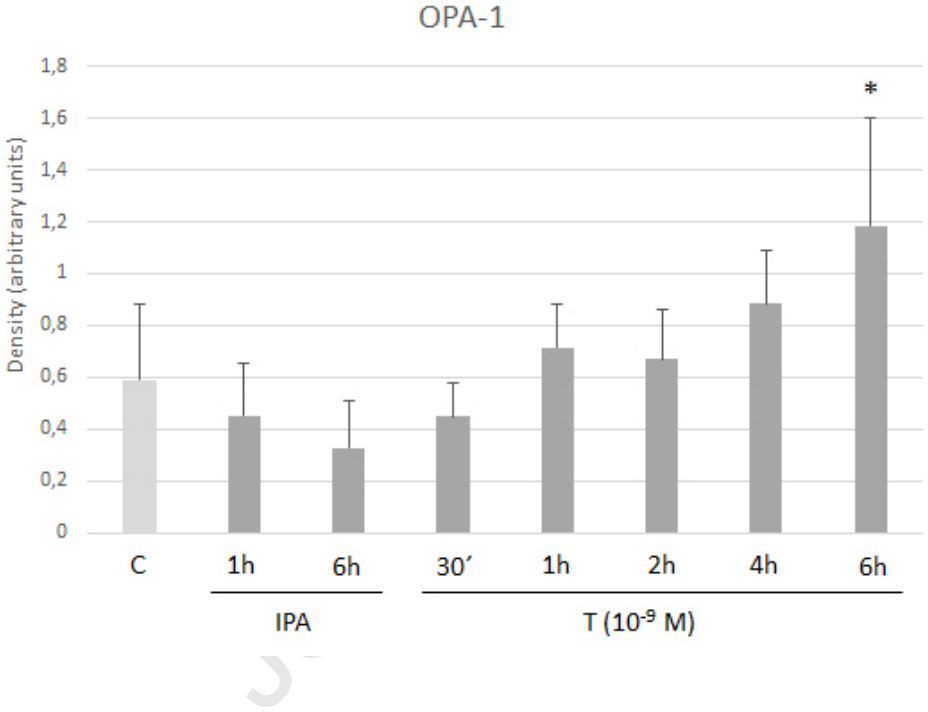

ND1
Relative mRNA ratio of ND1/GAPDH
120
100
80
60
40
20
0
C
30´
1h
3h
4h
H2O2


ND4
Relative mRNA ratio of ND4/GAPDH
120
100
80
60
40
20
0
C
30´
1h
3h
4h
H2O2

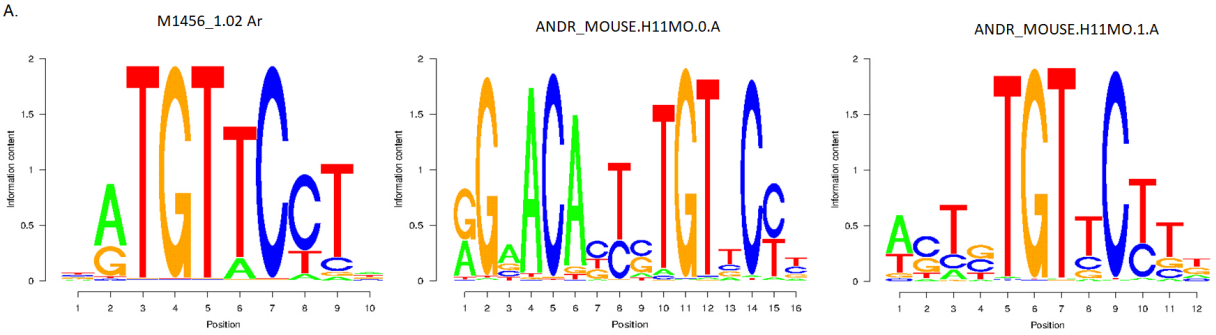

Cyt B
Relative mRNA ratio of CytB/GAPDH
120
100
80
60
40
20
0
C
30´
1h
3h
4h
H2O2


COX1
Relative mRNA ratio of COX1/GAPDH
300
250
200
150
100
50
0
C
30´
1h
3h
4h
T
T + F
*
#
COX2
Relative mRNA ratio of COX2/GAPDH
350
300
250
200
150
100
50
0
ND1
Relative mRNA ratio of ND1/GAPDH
*#
ND4
Relative mRNA ratio of ND4/GAPDH
Cyt B
Relative mRNA ratio of CytB/GAPDH

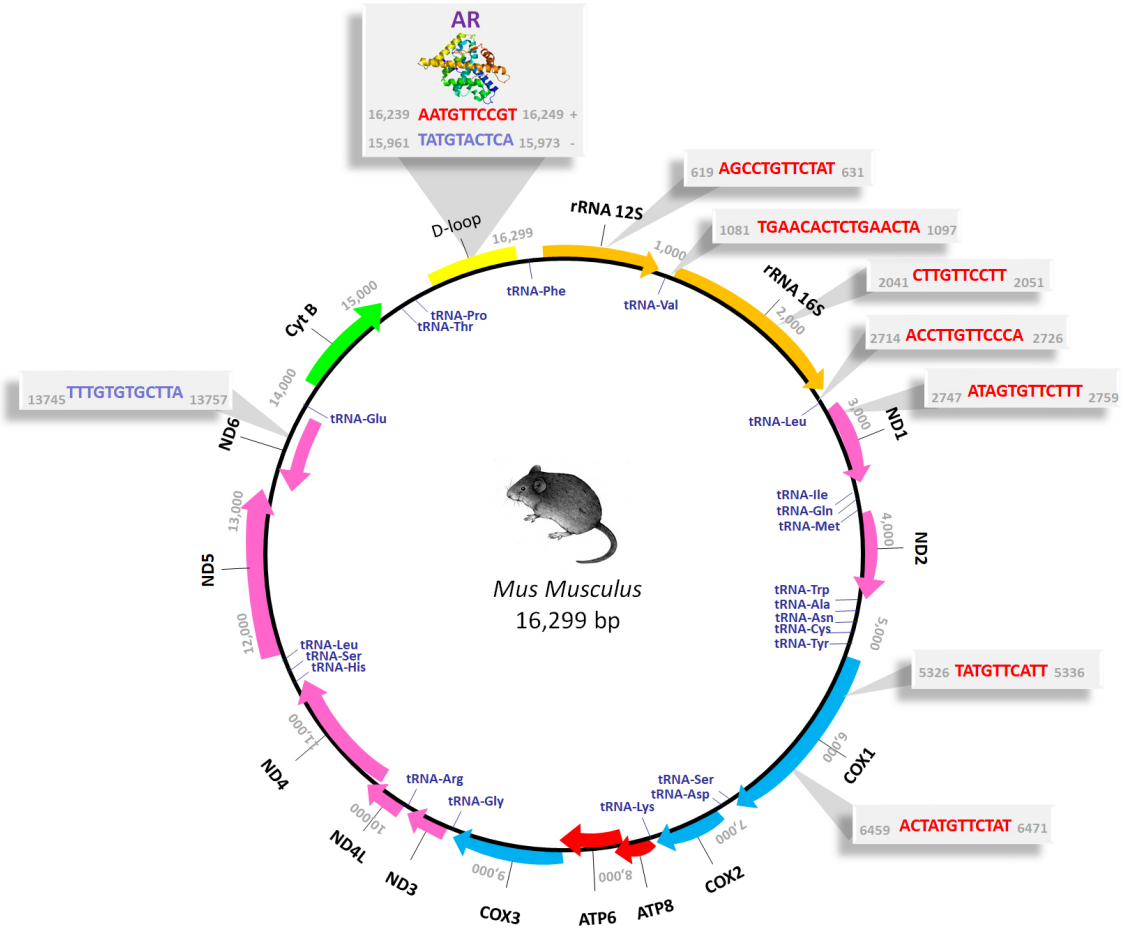

IPA (0.001%)
T (10-9 M)
C
1h
6h
30´
1h
2h
4h
6h
OPA-1
β-Tubulin


OPA-1
Density (arbitrary units)
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
C
1h
6h
30´
1h
2h
4h
6h
IPA
T (10-9 M)

A.


M1456_1.02 Ar
ANDR_MOUSE.H11MO.0.A
ANDR_MOUSE.H11MO.1.A
Information content
Position

B.


AR
16,239 AATGTTCCGT 16,249 +
15,961 TATGTACTCA 15,973 -
619 AGCCTGTTCTAT 631
1081 TGAACACTCTGAACTA 1097
2041 CTTGTTCCTT 2051
2714 ACCTTGTTCCCA 2726
2747 ATAGTGTTCTTT 2759
5326 TATGTTCATT 5336
6459 ACTATGTTCTAT 6471
13745 TTTGTGTGCTTA 13757
D-loop
rRNA 12S
rRNA 16S
ND1
ND2
COX1
COX2
ATP8
ATP6
COX3
ND3
ND4L
ND4
ND5
ND6
Cyt B
tRNA-Phe
tRNA-Val
tRNA-Leu
tRNA-Ile
tRNA-Gln
tRNA-Met
tRNA-Trp
tRNA-Ala
tRNA-Asn
tRNA-Cys
tRNA-Tyr
tRNA-Ser
tRNA-Asp
tRNA-Lys
tRNA-Gly
tRNA-Arg
tRNA-His
tRNA-Ser
tRNA-Leu
tRNA-Glu
tRNA-Pro
tRNA-Thr
Mus Musculus
16,299 bp

Highlights

- Testosterone induces NRF1 and 2, Tfam and TFB2M transcription factors expression.
- Testosterone upregulates mitochondrial genes that encode MRC proteins.
- H_2O_2 downregulates mitochondrial genes that encode MRC proteins.
- Testosterone promotes mitochondria preservation by induction of OPA1 expression.
- Mouse mitochondrial genome contains putative AR binding sites.
- Testosterone modulates directly and indirectly mitochondrial gene expression.